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DEVELOPMENT OF AN RNA ASSAY TO ACCESS HIV-1 LATENCY

MIDTERM REPORT

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13. ABSTRACT *(Maximum 200 words)*

HIV-1 is the cause of a slowly progressive immunosuppressive disorder, characterized by a prolonged "latent" phase with low levels of virus replication, and a subsequent symptomatic phase with higher levels of virus replcation. Viral regulatory factors, such as the virion infectivity factor (VIF), the trans-activator protein (TAT), the regulator of virus production (REV), of the negative factor (NEF) may play a role in determining the level of virus replication in vivo, and thus the progression to symptomatic disease. The current proposal is for continuation of DAMD contract for developing a diagnostic test with prognostic capabilities. The previous study was successful in the development of a sensitive, quantitative assay for HIV-1 DNS sequences. The current proposal is for the development of a complementary assay to hypothesis to examine specific HIV-1 RNA transcripts which may provide additional diagnostic utility. One hypothesis to be tested is that the presence or absence of mRNAs for VIF, TAT/REV, and/or NEF will be closely associated with the stage of clinical disease. Alternatively, the levels of these mRNAs or their predicted protein sequences will correlate closely with the stage of disease. The

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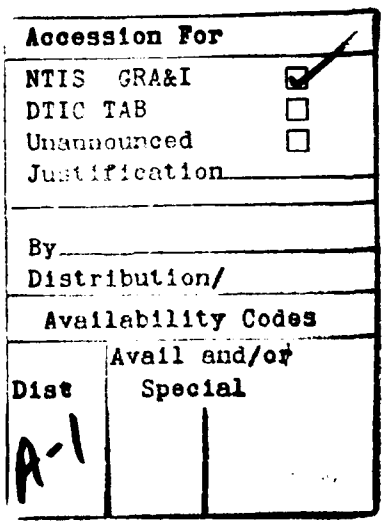
polymerase chain amplification reaction (PCR) will be applied for detection and quantitation of the specific viral RNAs from HIV-1 infected cell lines noted above, as well as several cellular mRNAs to serve as controls. The assay will then be applied to RNAs from peripheral blood leukocytes of patients at different stages of disease. Lastly, sequence data will be obtained for VIF, TAT, REV, NEF from some of these amplified sequences.



Summary

The goal of the current project is to develop a sensitive, quantitative RT-PCR assay to detect mRNAs for regulatory genes (vif, tat, rev, and nef) and a structural gene (env) in HIV-1 infected cell lines. In the first year of the project, we have synthesized all of the required reagents and established the optimal methods for collection of peripheral blood mononuclear cells and preparation of RNAs for this project. We have developed sensitive PCR assays for env as well as gag-pol transcripts. Several modifications of the originally proposed methods have been introduced to overcome difficulties in identifying mRNAs for regulatory genes.

FOREWORD

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TABLE OF CONTENTS

Specific Aims

1. To develop a sensitive PCR assay to detect mRNAs for regulatory genes (vif, tat/rev, and nef) and a structural gene (env) in HIV-1 infected cell lines

2. To validate the assay using RNA from cells transfected with HIV-1 proviral mutants or a cDNA clone

3. To amplify sequences from a number of cellular or synthetic mRNAs to serve as reference standards for the efficiency of the assay.

4. To apply the assay to peripheral blood leukocytes and other tissues of HIV-1 infected patients at different stages of disease for detection of HIV-1 vif, tat, rev, nef, and env mRNAs in comparison to selected reference RNAs.

5. To quantitate the levels of each of the RNAs in cell lines and patient samples

6. To sequence amplified products of vif, tat, rev, and nef to determine if abnormalities of the protein products are associated with disease progression

Hypothesis:

Viral regulatory gene products, vif, rev, tat, and/or nef are critically involved in determining the progression of clinical disease in HIV-1 infected individuals. We hypothesize that the presence or absence of mRNAs for one or more of these viral proteins, the levels of the mRNAs, or the predicted protein sequences will correlate closely with the stage of infection. These data are likely to be useful in enhancing our understanding of the pathogenesis of HIV-1 infection and in providing an assay capable of predicting which individuals are progressing to more profound stages of immunosuppressive disease.

Background and Signficance

Human immunodeficiency virus type 1 (HIV-1) is the cause of a slowly progressive immunosuppressive disorder, the acquired immunodeficiency syndrome (AIDS) (Wong-Staal & Gallo, 1985). Though at least 50% of infected individuals ultimately develop AIDS or AIDS-related complex (ARC), a long asymptomatic period is often present lasting from a few months to more than 5 years (Moss et al., 1988). Levels of active virus replication are thought to be significantly lower during the asymptomatic period of infection than during ARC or AIDS (Allain et al., 1987). Thus, the virus is described as "latent" during the asymptomatic stage of disease.

The state of virus replication during the clinically "latent" phase remains to be characterized. It is unclear whether viral transcription, translation, post-translational modifications, assembly, release, and/or dissemination of virus is restricted during this phase of disease. There is evidence that in lentivirus model systems like visna, transcriptional regulation plays a critical role in controlling latency (Haas, 1986).

A latent stage of virus replication can also be developed in tissue culture model systems (Folks et al., 1986; Folks et al., 1987; Zagury et al., 1986). After infection of primary lymphocytes or certain lymphoid or monocytoid cell lines, the majority of cells give rise to replicating HIV-1, and undergo cytopathic effects. However, a subpopulation may escape cell killing and remain viable for prolonged periods of time. Though HIV-1 provirus can be identified in these cells, expression of viral gene products is not seen. Activation of these cells with non-specific mitogens, antigens, or modified nucleosides results in the activation of viral gene expression and subsequent cell killing. Similar events have been hypothesed to be critical to the conversion of the latent provirus to actively expressed provirus in vivo.

A number of factors may be critical to the activation of HIV-1 from a latent state in vivo. These include secondary factors including antigenic stimuli from opportunistic infection (Zagury et al., 1986), transcriptional activation by DNA viruses or human T lymphotropic virus type I (HTLV-I) (Mosca et al, 1987; Siekevitz et al., 1988; Tong-Starksen et al., 1987), or DNA damage as may occur with ultraviolet light (Valerie et al., 1988). Host factors may play a role and these may include cellular regulatory factors as well as immunological regulators based on B or T lymphocytic functions. Lastly HIV-1 genetic regulation may also play a role in the level of replication at different stages of disease in vivo.

HIV-1 regulatory genes have been characterized in tissue culture systems but their role in vivo remains obscure. The vif gene is crucial for virion infectivity (Fisher et al., 1987). The tat gene product is a potent transactivator of viral gene expression, but its mechanism of action is not clear (Fisher et al., 1986; Dayton et al., 1986). Regulation may occur at the level of transcription, splicing, nuclear-to-cytoplasmic transport, RNA stability, or translation (Muesing et al., 1987; Rosen et al., 1986). The rev gene product is a differential regulator of viral protein synthesis (Sodroski et al., 1986; Feinberg et al., 1986). It functions at a post-transcriptional level, either at the level of splicing, nuclear-to-cytoplasmic transport, RNA stability, or translation. It acts to increase the levels of structural proteins as well as the pol products, whereas the levels of the regulatory products are decreased. The nef gene product is a negative factor that depresses viral transcription (Terwilliger et al., 1986; Luciw et al., 1987; Ahmad & Venketesan, 1988; Neiderman et al, 1989). The functions of the vpr and vpu genes remain to be determined.

Down-regulation of NEF, or up-regulation of VIF, TAT, and/or REV may be important for the activation of HIV-1 from a state of latency to actively replicating virus. Thus, by determining their levels at different stages in vivo, a better understanding of latency and disease progression should be developed. Current assays are unable to detect the levels of these gene products in tissues from infected individuals, due to a) the low level of expression of these gene products in the infected cells (Fisher et al., 1987, personal communication with F. Wong-Staal) and b) the small proportion of infected cells in any given tissue (Harper et al., 1986). A very sensitive technique, the polymerase chain amplification reaction, has now been developed which allows the detection of rare RNA or DNA sequences (Saiki et al., 1985; Saiki et al., 1988). This technique will be used first for analysis of fresh tissues of HIV-1 infected patients for the presence or absence of vif, tat, rev, and nef mRNAs, for their quantitation, and to provide amplified sequences to determine the predicted protein products.

Experimental Methods and Results

i) To develop a sensitive PCR assay to detect mRNAs for regulatory genes (vif, tat/rev, and nef) and a structural gene (env) in HIV-1 infected cell lines

The HIV-1 genome can be transcribed into an unspliced mRNA (for GAG and GAG-POL), singly spliced mRNAS (for ENV, VPU, and ? VIF and ? VPR), and multiply spliced mRNAs (for REV, TAT, NEF, and ? VPR, and VIF). A number of splice donor and acceptor sites have been mapped by sequencing cDNA clones of HIV-1 (Muesing et al., 1985; Arya et al., 1986) and by S1 nuclease mapping (Sadaie et al., 1988; Ratner and Okamoto, unpublished findings). These are shown schematically in Figure 1. All spliced mRNAs utilize the same splice donor site at position 287 (SD1). All multiply spliced mRNAs utilize the same splice donor site at position 5625 (SD2C) and the same splice acceptor site at position 7955 (SA2C). Less commonly variants to SA2 are utilized which are at position 7932 (SA2B) or at position 7927 (SA2A).

The various HIV-1 genes utilize different additional splice donors (SD2A, SD2B) at positions 4542 and 5043, respectively, and splice acceptors (SA1A, SA1B, SA1C, SA1D, SA1D', and SA1E), at positions 4492, 4969, 5356, 5530, 5532, and 5556, respectively, in various combinations. All possible mRNAs utilizing these combinations of splice donors and splice acceptors are shown in Figure 1. Several of these mRNAs have been demonstrated to exist by analysis of cDNA clones. Examination of the sequences between nucleotides 4492 and 5625 fail to demonstrate other consensus splice donor and acceptor sites. Furthermore, S1 mapping data with sequences from this portion of the viral genome have also failed to identify additional splice donor or acceptor sites (Ratner and Okamoto, unpublished).

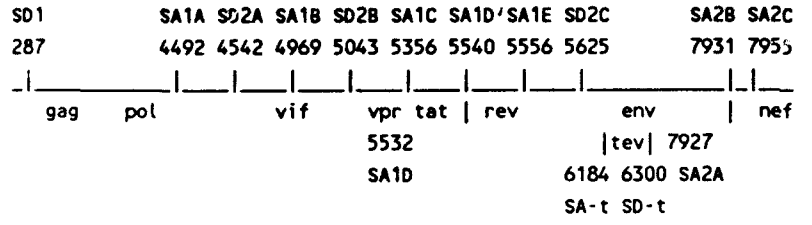
One can identify the protein encoded by each mRNA assuming that the first AUG codon in the mRNA is recognized by the ribosome. Examples of utilization of downstream initiator AUG have been presented in the literature (Ratner et al., 1987; Fink, 1986; Kozak, 1986), but this is not common. Even if some of the mRNAs shown in Figure 1 actually encode additional proteins, it is likely that the first AUG codon will be used predominantly. Correlations will be made with specific mRNAs and stage of disease, and do not depend entirely on our assignment of the coding potential of individual mRNAs. If critical mRNAs are identified which are expressed at different levels at different disease stages, further work will examine the coding potential of cDNAs corresponding to those mRNAs in eukaryotic systems with the use of specific antisera, but these studies will be outside the scope of this proposal.

All of the primers described in Fig. 1 and Table 1 for identification of transcripts for regulatory mRNAs have been synthesized during the first year of this study. In addition, we have synthesized several other oligonucleotides as described in Fig. 2. These include oligonucleotides SK37, JA4, SK145, and SK101 in the gag gene; JA17, JA20, JA18, and JA19 in the pol gene; and SK68 and JA14 in the env gene. Furthermore we have synthesized LA41v, an oligonucleotide just downstream of SA2C. Closely situated oligonucleotide primers to LA41v which have been synthesized include LA41T, LA42, and LA43 which may overcome problems with sequence heterogeneity to the LA41v annealing site. Lastly we have synthesized primers that flank the predominantly utilized SD2C/SA2C sites that are utilized by transcripts for all regulatory proteins.

For amplification of vif, tat, rev, and nef mRNAs we have used a primer (S2) which is complementary to nucleotides 8666-8641, a well conserved sequence (Myers et al., 1988) downstream of SA2 and within the polypurine tract was used to prime (+) strand synthesis (Table 1). To synthesize the complementary DNA strand, 100 pmoles of S2 wee added to 1 microgram of RNA together with 200 units of MuLV RT, 1 mM of each dNTP, and the buffer specified by the manufacturer (50 mM Tris-Cl, pH 8.3, 8 mM magnesium chloride, 10 mM dithiothreitol), in a final volume of 20 microliters. The sample was incubated at 37 degrees C. for 30 minutes, and then at 94 degrees C. for 1.5 minutes. However, in work performed this year, we also analyzed priming with oligo dT or random hexamers, and found the greatest efficiency was with the use of random hexamers.

For the subsequent PCR reactions, 100 pmoles of primer S1 was added which is identical to nucleotides 228-250, which are conserved in all known HIV-1 isolates and are located just upstream of SD1. Primer S2 was added when not used in the reverse transcriptase step. In addition, 8 microliters of 10 X Taq polymerase buffer (500 mM KCl, 100 mM Tris-Cl, pH 8.3, 15 mM magnesium chloride, 0.1% gelatin) was added with 2.5 units Taq polymerase (Cetus) and water to 100 microliters. The samples were then placed on the thermocycler (Perkin-Elmer-Cetus) for 94 degrees C for 1.0 min., 37 degrees C for 2.0 min., and 72 degrees C for 10 min. for a total of 30 cycles for RNA from HIV-1 infected H9 cells. RNA from uninfected H9 cells was used as a negative control.

Figure 1 - Schematic of HIV-1 Genome and Positions of SD and SA Sites:



Sequences Within Each Possible mRNA								Reactivity with LA45/LA41 \ Protein	Oligo Reactivity	Size of PCR Product	Clone Isolated	Reference
Sequences Amplified With S1 and S2:								\				
gag-pol	vif	vpr	tat	rev	env	nef						
sd1	sd2a	sd2b			sd2c	sd-t						
	sa1a	sa1b	sa1c	sa1d	sa1e	sa-t	sa2					
-	----------						---- +	VIF	S3,S-VIF	1906		Arya et al., 198
-	----------	----------					---- +	VIF	S3,S-VIF	1594		Schwartz et al.,
-	----------		---------				----	VIF	S3,S-VIF	1420		
-	----------			----			----	VIF	S3,S-VIF	1394		
-	----	----------					---- +	VPR		1480		
-		----------					---- +	VPR		1429		Schwartz et al.,
-	----	----	----------				---- +	TAT	S6	1168		
-		----	----------				---- +	TAT	S6	1117	H9c181	Muesing et al.,
-	----		----------				---- +	TAT	S5	1093	H9c177	Muesing et al.,
-			----------				---- +	TAT	S4	1042	H9c171, pCV1	Muesing et al.,
												Arya et al., 198
-	----	----	----------		---		---- +	TEV	S6,TEV1,TEV2	1285		
-		----	----------		---		---- +	TEV	S6,TEV1,TEV2	1234		
-	----		----------		---		---- +	TEV	S5,TEV1,TEV2	1210		
-			----------		---		---- +	TEV	S4,TEV1,TEV2	1159		Schwartz et al.,
-	----	----		---------			----	REV/NEF	S-REV3	984,992		
-		----		---------			----	REV/NEF	S-REV3	933,941		Schwartz et al.,
-	----			---------			----	REV/NEF	S-REV2	909,917		Schwartz et al.,
-				---------			----	REV/NEF	S-REV1	858,866		Schwartz et al.,

-	----	----	---------	---	-- -			1101,1109		
-		----	---------	---	----			1050,1058		
-	----		---------	---	----			1026,1034		
-			---------	---	----			975,983		Schwartz et al.,
-	----	----	----		----	NEF	S9	992		
-		----	----		----	NEF	S9	917		Schwartz et al.,
-	----		----		----	NEF	S8	893	H9c176	Muesing et al.,
-			----		----	NEF	S7	842	pCV3	Arya et al., 198
-	----	----	----	---	----		S9	1169		
-		----	----	---	----		S9	1034		
-	----		----	---	----		S8	1010		
-			----	---	----		S7	959		Schwartz et al.,

* Utilization of SD2A or SD2B rather than SD2C will lead to identical PCR products but which are 25 or 29 nt larger
* Utilization of LA8/S11 yields PCR products that are 28 bp shorter than listed above S1/LA41 yields PCR products that are 670 nt sho LA8/LA41 yields PCR products that are 698 nt shorter
* Utilization of LA45/LA41 yields PCR products which are 132 nt in length ± 117 nt if tev exon included (+ in table indicate with these primers

Sequences Amplified With S1 and S10:

-	----	----	----------	VPU/ENV	782	
-		----	----------	VPU/ENV	731	
-	----		----------	VPU/ENV	707	
-			----------	VPU/ENV	656	Muesing et al., 1985
-	----	----	----------	Aber. REV	808	
-		----	----------	Aber. REV	757	
-	----		----------	Aber. REV	733	
-			----------	Aber. REV	682	
-	----	----	----------	Aber. TAT	932	
-		----	----------	Aber. TAT	931	
-	----		----------	Aber. TAT	907	
-			----------	Aber. TAT	856	
-	----	----------		VPR	1294	
-		----------		VPR	1243	
-	----------		----------	VIF	1208	
-	---------- ---		----------	VIF	1234	
-	----------	----------		VIF	1408	
-	----------			VIF	1720	

Table 1 - Primers and Probes Used in HIV-1 RNA PCR

Primer	HIV-1 Sequences	Sequence
S1	228- 250	TCTCTCGACGCAGGACTCGGCTTGC
S2	8666-8641	GCCAGTCCCCCCTTTTCTTTTAAAAA
S10	6151-6124	TTTGATCCCATAAACTGATTA
		G
S1-Eco	228-250	TTCCGAATTCTCTCGACGCAGGACTCGGCTTGC
S2-Eco	8666-8641	TTCCGAATTCGCCAGTCCCCCCTTTTCTTTTAAAAA

Probe	HIV-1 Sequences	Sequence	Presumed Protein Product	PCR Product Size With S1,S2 Primers	With S1,S10 Primers
S3	4901-4920	ATAGCACACAAGTAGACCT	VIF	1906	1720
			VIF	1594	1408
			VIF	1420	1234
			VIF	1394	1208
S-VIF	4621-4660	ATGGAAAACAGATGGCAGGTGATGATTGTG	As above for S3		
S4	SD1/SA1C	GGCGGCGACTG/AATTGGGTGTCGAC	TAT	1042	856
		A AA AC AG			
			TEV	1159	
S5	SD2A/SA1C	CTCTGGAAAG/AATTGGGTGTCGAC	TAT	1093	907
		AG			
			TEV	1210	
S6	SD2B/SA1C	GGACATAACAAG/AATTGGGTGTCGAC	TAT	1117	931
		AG	TAT	1168	982
			TEV	1285	
			TEV	1234	
S-TEV-1	SD2C/SA-t	TGCACTTTAAATGCTTTGATAG	TEV	1285	
			TEV	1234	
			TEV	1210	
			TEV	1159	
				1101	
				1050	
				1026	
				975	
				1169	
				1034	
				1010	
				959	
S-TEV-2	SD-t/SA2C	GGGAGGTGGGTCTCTTATGCTT	Same as for S-TEV-1		

S13	SD1/SA1D	GGCGGCGACTG/GCATCTCCTATGGC	REV	868	682
		A AA AC			
S14	SD2A/SA1D	CTCTGGAAAG/GCATCTCCTATGGC	REV	919	733
S15	SD2B/SA1D	GGACATAACAAG/GCATCTCCTATGGC	REV	943	757
			REV	994	808
S7	SD1/SA1E	GGCGGCGACTG/GAAGAAGCGGAGA	NEF	842	
		A AA AC A	VPU/ENV		656
S8	SD2A/SA1E	CTCTGGAAAG/GAAGAAGCGGAGA	NEF	893	
		A	VPU/ENV		707
S9	SD2B/SA1E	GGACATAACAAG/GAAGAAGCGGAGA	NEF	917	
		A	NEF	992	
			VPU/ENV		782
			VPU/ENV		731
S10	6151-6124	TTTGATCCCATAAACTGATTA	VPU/ENV		782
		G	VPU/ENV		731
			VPU/ENV		707
			VPU/ENV		656
			REV		808
			REV		757
			REV		733
			REV		682
			TAT		982
			TAT		931
			TAT		907
			TAT		856
			VPR		1294
			VPR		1243
			VIF		1720
			VIF		1408
			VIF		1234
			VIF		1208

Table 2 - Amplified Products Expected from Cells Transfected with HIV-1 Proviral Mutants and a cDNA Clone

Clone	Deleted Sequences	PCR Product Sizes				
		Primers S1 and S2				Primers S1 and S10
		Probe S3	Probes S4-S6	Probes S13-S15	Probes S7-S9	Probes S7-S9
		VIF	TAT	REV	NEF	
HXB2gptX	-	1394	1042	868	842	656
		1420	1093	919	893	707
		1594	1117	943	917	731
		1906	1168	994	992	782
HXB2gptE	4348-5323	-	1042	868	842	656
HXB2gptdS	4702-5255	-	1042	868	842	656
			1093	919	893	707
MP76	4702-5255					
	5904-6122	-	1042	868	842	437^
			1093	919	893	488^
pCV1	cDNA clone	-	1042	-	-	-

^ shortened transcript due to deletion

Ten microliters of each PCR product are analyzed on a 5% polyacrylamide gel on a Hoefer minigel apparatus. Markers on the same gel will include 32P-end labeled Hae III Phi-X DNA fragments (labeled with polynucleotide kinase and 32P-gamma-ATP).

In the first year of this project, we had significant difficulties with "carry-over" contamination from amplified products into unamplified products. These problems have been solved by taking numberous precautions in addition to the use of a separate room for PCR reaction set-up and analysis and use of separate pipetters and reagents for pre-PCR and post-PCR steps. We have found that ultraviolet light irradiation of the buffers and nucleotide triphosphates descreased the incidence of contamination greatly. However, we also found that ultraviolet light caused diminution in Taq polymerase activity and primer efficiency.

We have devoted considerable effort in the first year of this study to optimizing the efficiency of the PCR methods. It was rapidly apparent that nested PCR techniques were required to obtain a highly sensitive technique capable of detecting 1-10^4 copies. We have optimized the magnesium concentration in both the first and second rounds of nested PCR to within a range of 1.5-7 mM, differing for each set of primers. We have determined the optimal amount of primer in each round of the reaction to be 60 ng (about 5 pmol), which is considerably lower than the amounts of primer we had originally planned to use. We have determined that 35 cycles in both the first and second rounds of nested PCR provides a highly sensitive assay without contamination.

To simplify the assays, rather than utilize hybridization, we have chosen to end-label with polynucleotide kinase one of the primers in the second set of nested PCR reactions. In the case of the transcripts for regulatory proteins, we utilize S1 and S2 in the initial PCR reactions, and LA41v (or LA41T, LA42, or LA43) with one of the specific primers flanking a splice site (i.e. S3, S4, S5, ...etc). Thus, we label LA41v, the primer shared in each case. Thus, amplified bands can be visualized on polyacrylamide gels, by drying the gel and exposing to xray films for 1-24 hrs.

A significant advantage of the methods proposed here is that HIV-1 DNA sequences will not be amplified by the primers chosen since the products would be too large to be amplified efficiently. We have succeeded in amplifying sequences of up to 2 kb from both the HIV-1 env gene and from the c-sis gene, but larger sequences could not be amplified efficiently. Thus, only HIV-1 RNA sequences and not HIV-1 DNA sequences contaminating the RNA preparations will be amplified. The one exception may be that defective HIV-1 proviruses could potentially be amplified by the scheme outlined above.

In the initial studies we have utilized total RNA prepared from uninfected and HIV-1 infected H9 cells using the guanidine isothiocyanate procedure of Chirgwin (1979). However, we found a simpler and more efficient mode of RNA preparation from clinical samples to be with the Fast-Trak system which provides poly A+ RNA.

During the first year, we have completed standardization of the reaction conditions with plasmid DNAs. We have isolated the necessary cell line RNAs (from H9 and HIV1hxb2 infected H9 cells) and RNAs from primary mononuclear cells from five patients. In addition we have synthesized from the riboprobe vector a tat RNA which will be utilized in standardization of the reaction conditions.

ii) <u>To validate the assay using RNA from cells transfected with HIV-1 proviral mutants and a cDNA clone</u>

We will use particular HIV-1 proviral mutants that we have constructed to confirm our hypotheses about the nature of the mRNA species detected. The parental virus is HXB2gptX which includes the entire HIV-1 genome. It is expected to give all possible size classes of mRNAs if indeed they are actually expressed (Table 2). Cells transfected with each of four different proviral mutants or a cDNA clone will be used as sources of RNAs for comparison. HXB2gptE is derived from HXB2gptX by removal of the 1.0 kb Eco RI fragment (nucleotides 4348-5323) which includes the 3' portion of pol, vif, most of vpr and SA1A, SD2A, SA1B, and SD2B. RNA from cells transfected with this clone should include no vif mRNA, only the tat mRNA which gives an amplified product of 1042 nucleotides, only the rev mRNA which gives an amplified product of 868 nucleotides, only the nef mRNA which gives an amplified product of 842 nucleotides, and the env mRNA which gives an amplified product of 656 nucleotides.

Clone HXB2gptdS is derived from HXB2gptX by removal of a 0.8 kb fragment (nucleotides 4702-5255) between Nde I and Nco I sites which includes a portion of vif and vpr, and SA1B and SD2B. RNA from cells transfected with this clone should include a vif mRNA which will be lacking sequences capable of hybridizing with probe S3, tat mRNAs which give amplified products of 982 and 1033 nucleotides, rev mRNAs which give amplified products of 807 and 919 nucleotides, nef mRNAs which give amplified products of 782 and 893 nucleotides, and env mRNAs which gives amplified products of 656 and 707 nucleotides.

Clone MP76 is derived from HXB2gptdS by digestion with Nde I and Bal 31 treatment removing sequences 5904-6122. RNA from cells transfected with this clone should give the same size amplified products described above from clone HXB2gptdS but the PCR product derived from env mRNA should be only 437 and 488 nucleotides due to the second deletion.

Clone pCV1 (kindly provided by F Wong-Staal, NCI) is a cDNA clone of the tat mRNA expected to give the 1042 nucleotide amplified product. Thus, RNA from cells transfected with this clone should give rise to only that amplified product.

RNA will be obtained from 150 mm plates of COS-1 cells transfected with 20 micrograms of each of the above proviral DNAs at 50% confluence and then grown for 3-4 days. Cells will be scraped and RNA isolated by the method of Chirgwin, as described above. We have previously demonstrated that each clone noted above (except pCV1) is capable of giving rise to significant levels of soluble p24 antigen in the medium, and are thus actively expressed clones. Clone pCV1 has been shown to be active by its ability to trans-activate HIV-1 LTR-CAT. COS-1 cells transfected with a control plasmid lacking HIV-1 sequences will be used as a negative control in these experiments.

These experiments should confirm that the size classes of each different mRNAs correspond to those which are predicted by the scheme in Figure 1. If differences arise from those which are predicted, additional mutant proviruses will be studied to clarify these questions.

Each of these proviral clones have been prepared and tested.

iii) <u>To amplify sequences from a number of cellular or synthetic mRNAs to serve as reference standards for the efficiency of the assay</u>

The failure to amplify a particular HIV-1 RNA sequence may be due to its absence, its low level in the infected cell, poor sample preparation due to degradation of RNA, or due to a contaminant in the preparation that inhibits the RT or Taq polymerase reactions. It should be recognized that this method is not highly dependent on having fully intact RNAs, but RNAs degraded to fragments of less than 1-2 kb are not likely to serve as efficient substrates for PCR. Thus to exclude the latter two artifacts we will also amplify sequences from endogenous cellular sequences or added synthetic RNA sequences. These sequences may also serve as a reference standards for quantitation.

The choice of an endogenous cellular sequence to use as a reference standard is difficult. One would like to use a transcript whose level is independent of the presence of HIV-1 infection, CD4 depletion, stage of HIV-1 disease, state of lymphocyte or monocyte differentiation, or presence or absence of secondary opportunistic infection or neoplasms. We have chosen three transcripts with which we have had considerable experience and have thus far not found to be affected by at least some of these parameters. More experiments are required to validate this assumption. The transcripts chosen are indicated below together with the primers and probes utilized in each case.

a) human beta cytoplasmic actin (Ng et al., 1985)

primers:

ACTIN K: CCAACCGCGAGAAGATGACCCAG (nucleotides 1631-1653 in exon 3)
ACTIN L: GCCGGGAGACAGICTCCACTCAC (nucleotides 2556-2534 in exon 5)

probe:

ACTIN M: CCCAAGACCCCAGCACACTTAGCCGT (nucleotides 1981-2006 in exon 4)

b) aldolase A

primers:

Df2: CTCCATCAATGAACTGCCAAATGA (nucleotides 1194-1217)
Dr3: CATCAAATGCACTATTCTCAACAC (nucleotides 1017-1040)

probe:

Dr4: AAGAGCTATGCCTACACACAGGCTG

c) Alu I sequences (derived from sequences within the 5th c-sis intron [Smidt et al., 1989])

primers:

SIS S: GGCTGGGCGTGGTGGCTCACGCC (nucleotides 405-427)
SIS T: AATTTTTGTATTTTTAGTAGAGA (nucleotides 674-652)

probe:

SIS U: GCCTGTAATCCCAGCACTTTGGGAGGCC (nucleotides 560-587)

In addition, we will explore the usefulness of adding a synthetic mRNA to the isolated cells that are used for RNA preparation. We have chosen c-sis sequences since these are known not to be expressed in peripheral blood leukocytes except from activated monocytes, and because we have had extensive experience with studies of the c-sis gene and have already constructed the necessary vectors (Ratner et al., 1987). Even if endogenous c-sis sequences are found in the blood samples due to monocyte activation in vivo, these will be distinguished from the synthetic mRNAs which will include intron sequences as well, or can be distinguished by the use of antisense sequences. We have subcloned a 2.0 kb DNA fragment from portions of exons 6 and 7 and the intervening intron of the human c-sis gene into a plasmid containing an SP6 promoter in either orientation. We have succeeded in generating full length unlabeled transcripts with SP6 polymerase. Small amounts of these RNAs (1 pg - 1 ng) will be added to cells from HIV-1 infected cell lines or from peripheral blood leukocytes prior to preparation of RNA by the method chosen above. The relevant sequences will be amplified with the following primers and detected with an end-labeled oligonucleotide probe based on intron sequences which will be determined by nucleotide sequencing.

synthetic c-sis RNA primers (based on sequences in Josephs et al., 1984):

SIS V: CCCTCCACGTGTCCGTCCACCCTTCCATCAG (nucleotides 990-1020)
SIS W: CAAGACCGGGAGGCCGCTGGGAGGAGACCCG (nucleotides 1050-1021)

One or more of the most suitable sequences chosen from studies above with uninfected and HIV-1 infected cell lines and then a limited number of patient samples will be utilized for studies with all patient samples.

During the first year, all of the primers listed in this section have been synthesized.

iv) To apply the assay to peripheral blood leukocytes and other tissues of HIV-1 infected patients at different stages of disease for detection of HIV-1 vif, tat/rev, nef, and env mRNAs in comparison to selected reference RNAs

During the first year of the study, we have obtained 10-40 ml of blood for RNA preparation by the simplest effective method as described in section i.

Ten microliters of each RNA sample will be used for PCR reaction. Samples from uninfected individuals, from HIV-1 infected cell lines, and uninfected cell lines have also been prepared. The same amplification techniques described above will be performed with these RNA preparations.

Conclusions

During the first year of the study, the necessary oligonucleotides were synthesized, proviral clones constructed, and RNAs purified.

The first year of this study has established the conditions to maximize the sensitivity and specificity of the RNA PCR methodology as applied to HIV1 for examining transcripts of structural and regualtory proteins. We have eliminated contamination with careful technique and ultraviolet irradiation of buffers. We have optimized magnesium concentration, primer concentration, and cycle number in the PCR. We have found that nested PCR techniques are required to acheive the sensitivity desired for this study.

Recommendations

During the second year of the study we will apply the PCR techniques for gag-pol, env, and tat mRNAs to RNAs from H9 and HIV1hxb2 infected H9 cells. Various titrations of plasmid DNA will be examined in the same experiments. If PCR is unsuccessful, we will examine PCR reactions with riboprobe synthesized transcripts corresponding to each mRNA and determine the number of RNA copies required to obtain positive reactions. Further optimization of reverse transcriptase reactions and PCR would then be carried out.

If PCR is successful with the tat primers, we will then attempt to amplify rev, nef, vif, and tev, with the primers described.

If successful, we will also perform PCR with a small number of RNA preparations from clinical samples to determine if the range of HIV1 sequences corresponds to that for which the assay was established.

We will explore the efficiency of the reactions with primer SD2/SA2Down and primer LA41v to determine the total concentration of transcripts for regulatory proteins. In addition we will utilize primer SD2/SA2Up in place of LA41v with specific primers for each regulatory protein transcript to increase the efficiency of the PCR reaction by generating a smaller PCR product.

We will then confirm the identity of the PCR products by PCR amplification from RNA samples of cell lines transfected with specific proviral mutants.

We will next assess our ability to PCR sequences from cDNA transcripts corresponding to actin, aldolase, IL2, IL2 receptor, or c-sis transcripts as described above. This will serve as a reference standard for all quantitation experiments.

We will then go on to apply our assay to clinical samples obtained from patients at different stages of disease. Selected transcripts will be cloned and sequenced to determine if structural alterations have occurred in particular gene products associated with disease progression.

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Manuscripts in preparation, submitted, or published from this work

None